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Genetic and biochemical studies indicate that the bacterial stress response protease HtrA represents an important defense against oxidative damage. More importantly, many facultative intracellular pathogens rely upon the HtrA to help them resist killing by the oxidative killing pathways of host macrophages. Studies completed to date under this contract indicate that although the *Brucella* HtrA contributes to resistance to oxidative killing *in vitro* and resistance to killing by cultured murine neutrophils and macrophages, *Brucella htrA* mutants are not attenuated in the murine host. Attempts to combine *htrA* mutations with other mutations predicted to attenuate the brucellae have also met with limited success. During the course of these studies, however, we have identified five other genetic loci, *hfq*, *bacA*, *katE*, *clpA* and *uvrA*, which offer considerable promise as targets for the construction of novel *Brucella* vaccine candidates. We plan to continue investigating the nature of the attenuation of the corresponding *Brucella* mutants as well as examining the potential of these mutants to elicit protective immunity in the mouse model.

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FOREWORD

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R. M. Roop II 8/30/99

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Brucellosis is a serious zoonotic disease (Acha and Szyfres, 1980) which poses a potential threat to military personnel stationed in many areas of the world. These individuals are at risk either through accidental exposure to infected animals or animal products (Nicoletti, 1980) or through deliberate exposure to *Brucella* spp. as a component of biological warfare by an opposing military force (Huxsoll et al., 1987; Kaufmann et al., 1997). Unfortunately, there is currently no safe, effective brucellosis vaccine for use in humans. Prolonged survival and replication in host macrophages is a key component of disease production by *Brucella* spp. (Baldwin and Winter, 1994), and current evidence indicates that reactive oxygen intermediate (ROI)-mediated killing is the primary mechanism by which host macrophages eliminate intracellular brucellae (Jiang et al. , 1993). Therefore, cellular components which contribute to the resistance of the brucellae to oxidative killing by host phagocytes represent important virulence determinants. Biochemical and genetic studies have clearly shown that bacterial stress response proteases of the high temperature requirement A (HtrA) family have the capacity to degrade oxidatively damaged proteins before they accumulate to toxic levels within cells (Davies and Lin, 1988; Johnson et al., 1991; Li et al., 1996). In performing this function, members of this particular class of periplasmic serine proteases serve as an important secondary line of defense against ROI-mediated killing. Genetic studies in *Brucella abortus* and *Brucella melitensis* have confirmed the participation of the HtrA protease in cellular defense against oxidative damage (Elzer et al., 1994; Phillips et al., 1995; Robertson et al. 1996), and further suggest that this protease contributes to the survival and replication of these facultative intracellular pathogens in host macrophages (Elzer et al., 1996b) and their virulence in natural and experimental hosts (Edmonds et al., 1997, Elzer et al., 1994; Elzer et al. 1996a; Elzer et al. 1996b, Phillips et al. 1995; Phillips et al. 1997, Robertson et al. 1996). *Salmonella* strains carrying *htrA* mutations have been used successfully as vaccines in both mice (Chatfield et al., 1992) and humans (Tacket et al., 1997), and evidence recently obtained with pregnant goats suggests that *Brucella htrA* mutants also hold promise as vaccine candidates (Phillips et al., 1997). Unfortunately, the attenuation of *Brucella htrA* mutants in mice is limited to the early stages of infection (Elzer et al. 1996b; Phillips et al. 1995), and this residual virulence has prevented their evaluation as experimental vaccines in this important model of human brucellosis. Therefore, the objective of the proposed studies outlined in the Statement of Work for contract DAMD17-98-C-8045 was to introduce secondary mutations into *B. abortus* and *B. melitensis htrA* mutants which will enhance and stabilize the attenuation of these strains in BALB/c mice. Because of the demonstrated importance of oxidative killing in the elimination of intracellular brucellae from host neutrophils and macrophages, our initial experimental strategies focused on introducing mutations predicted to further compromise the already defective capacity of *Brucella htrA* mutants to resist ROI-mediated killing by host phagocytes. The first set of mutations introduced into the *Brucella htrA* mutants targeted the genes encoding the primary antioxidants catalase (*katE*) (Sha et al., 1994) and Cu-Zn superoxide dismutase (*sodC*) (Bricker et al., 1990). Because these proteins directly participate in the detoxification of H_2O_2 and O_2^-, KatE and SodC deficient strains of *Brucella* should accumulate abnormally high levels of these reactive oxygen intermediates (ROIs). This in turn is expected to lead to an increased level of oxidative damage to cellular proteins, thereby exploiting the cell's lack of a functional HtrA. The results of these experiments are detailed in this report. The second type of mutation introduced into the *Brucella htrA* mutants will target the *prc* gene, which encodes another stress response predicted to function in the elimination of ROI-damaged proteins from the periplasmic space (Davies and Lin, 1988; Bäumler et al., 1994). The loss of both of these proteases is predicted to severely hamper the capacity of the cell to deal with oxidative damage to its cellular proteins. These studies have recently been initiated.

BODY

I. Discovery of double mutant nature of *B. abortus* PHE1 and construction and characterization of authentic *B. abortus* and *B. melitensis htrA* mutants.

The strategy employed for the construction of the previously described *B. abortus htrA* mutant PHE1 (Elzer et al., 1994) involved replacing a segment of genomic DNA from virulent *B. abortus* 2308 encompassing the majority of the 5' end of the *htrA* gene and its promoter with a kanamycin resistance gene by allelic exchange (Figure 1). Nucleotide sequence analysis of the regions upstream of *htrA* revealed the presence of no open reading frames in this area and our subsequent genetic complementation of the *in vitro* and *in vivo* properties of PHE1 with a wild type *htrA* gene on a plasmid convinced us that the phenotype demonstrated by PHE1 was due to the *htrA* mutation alone. We have recently discovered the presence of a secondary mutation in PHE1, however, which was introduced inadvertently during the construction of this strain (R. W. Phillips and R. M. Roop II, manuscript in preparation). Just upstream of the *htrA* coding region lies the *cycHJKL operon*, which encodes genes involved in cytochrome c biosynthesis. The presence of these genes was first suspected when the close proximity of these same genes to *htrA* was reported in *Rhizobium meliloti* (Glazebrook et al., 1996). Upon re-examination of a larger region upstream of the *B. abortus htrA*, we discovered that a homologous *cycHJKL* operon was present in *B. abortus* 2308. Moreover, because the predicted *cycL* open reading frame overlaps the *htrA* promoter (Figure 1), the strategy employed to construct PHE1 truncated the *cycL* gene in addition to deleting the *htrA* gene. Thus, *B. abortus* PHE1, a strain originally described as an *htrA* mutant, is actually an *htrA cycL* double mutant. Accordingly, PHE1 gives a negative oxidase reaction *in vitro*, while the parental 2308 strain is oxidase-positive. The corresponding *B. melitensis htrA* mutant RWP5 (Phillips et al., 1995) was constructed employing the same gene replacement strategy as that employed for making PHE1. Therefore, based on the close genetic relatedness of *B. abortus* 2308 and *B. melitensis* 16M, and the observation that RWP5 is oxidase-negative while 16M is oxidase-positive, we are assuming that RWP5 is also an *htrA cycL* double mutant, although this has not been verified by nucleotide sequence analysis.

Even though strains PHE1 and RWP5 showed the phenotypic properties expected of *htrA* mutants, namely increased sensitivity to reactive oxygen intermediates and failure to form colonies on solid media at elevated growth temperatures (Elzer et al., 1994; Phillips et al., 1995), the phenotypes of these strains cannot reliably be used to evaluate the biological function of the *Brucella* HtrA due to the unknown contribution of the secondary mutation in *cycL*. Thus, a new mutational strategy, involving replacement of an internal portion of the *Brucella htrA* gene was used to construct *htrA* mutants derived from *B. abortus* 2308 (Figure 1) and *B. melitensis* 16M (data not shown). The genotype of the resulting mutants, *B. abortus* RWP11 and *B. melitensis* RWP21, has been verified by Southern blot analysis. *B. abortus* RWP11 is also oxidase positive (see above) and Western blot analysis has confirmed that this strain does not produce HtrA.

Consistent with the proposed biological function of the *Brucella* HtrA, the *B. abortus htrA* mutant RWP11 showed increased sensitivity to oxidative killing *in vitro* and susceptibility to the antibiotic puromycin (Table 1). Unlike 2308, this strain also showed growth restriction at elevated temperatures All three of these distinctive phenotypic properties were relieved by introduction of *htrA* into RWP11 on a pBBR1MCS-based plasmid vector. RWP11 also showed a significant decrease in its capacity to resist killing by both cultured murine macrophages (Figure 2) and neutrophils (Figure 3) compared to the parental 2308 strain. Although the sensitivity of this mutant to killing by cultured macrophages was equivalent to that observed for PHE1, RWP11 did not show the dramatic sensitivity to killing by neutrophils previously observed with the *htrA cycL* mutant (Elzer et al., 1996b). In this same vein, introduction of the *cycL* mutation alone into *B. abortus* 2308 (Figure 1) decreases the resistance of this strain to killing by macrophages and neutrophils *in vitro* (Figures 2 and 3). Again the intracellular survival profile of the *B. abortus cycL* mutant RWP501 was very similar to that of PHE1, but the defect imparted by this latter mutation with respect to killing by neutrophils was also much less severe than the defect observed previously in PHE1. Combined, these results strongly suggest that the extreme sensitivity of PHE1 to killing by murine neutrophils is caused by the

combination of the *cycL* and *htrA* mutations rather than by either mutation alone. To confirm this, we reconstructed a *B. abortus htrA cycL* mutant (Figure 1), designated RWP116, from virulent strain 2308 and examined the resistance of this strain to killing by cultured murine phagocytes (Figures 2 and 3). RWP116 demonstrated an identical phenotype in these studies to that described previously for PHE1 including the extreme sensitivity to killing by cultured neutrophils.

Despite their reduced capacity to resist killing by cultured murine phagocytes relative to 2308, neither RWP11 (2308 *htrA*) nor RWP501 (2308 *cycL*) was attenuated in BALB/c mice through 4 weeks post infection (Figure 4). This is in contrast to PHE1 (*htrA cycL*), which demonstrated significant attenuation limited to the early stages of infection (Elzer et al., 1996b). As expected, however, RWP116 (2308 *htrA cycL*) showed the *in vivo* phenotype described originally for PHE1 in mice, e.g. significant attenuation limited to the early stages of infection (Figure 4). These results indicate that the *in vivo* phenotype previously reported for PHE1 in mice was likely due to the presence of both the *htrA* and *cycL* mutations in this strain, rather than the presence of the *htrA* mutation alone. Correspondingly, the *B. abortus htrA* gene product does not appear to be essential for virulence in the murine model.

II. Evaluation of *B. abortus htrA, katE, sodC, htrA katE, htrA sodC* and *katE sodC* mutants in the BALB/c mouse model.

Although we have now established that the *htrA* mutation alone does attenuate *B. abortus* 2308 in BALB/c mice, this mutation does increase the sensitivity of 2308 to oxidative killing *in vitro* as well as impair its capacity to resist killing by cultured macrophages and neutrophils. To determine if the elimination of two important primary antioxidants, catalase (KatE) and copper-zinc superoxide dismutase (SodC) significantly enhances the impact of the *htrA* mutation on the virulence of 2308 in mice, *katE* and *sodC* mutations were introduced into RWP11. The *B. abortus htrA katE* mutant MEK14 was constructed employing allelic exchange to replace a 1 kb internal segment of the *katE* coding region in RWP11 with the chloramphenicol resistance gene from pBC. A similar strategy was used to replace a 136 bp region of the *sodC* gene in RWP11 with this same chloramphenicol resistance gene, resulting in the construction of MEK9. These same *katE* and *sodC* mutations were also introduced into *B. abortus* 2308, resulting in the construction of strains MEK 6 and MEK2, respectively. The genotypes of MEK14 (2308 *htrA katE*), MEK9 (2308 *htrA sodC*), MEK6 (2308 *katE*) and MEK2 (2308 *sodC*) were verified by Southern blot analysis with *htrA*-, *sodC*-, *katE*-, kanamycin resistance gene-, chloramphenicol resistance gene- and gene replacement vector-specific probes. The absence of SodC from MEK9 and MEK2 was also demonstrated by Western blot analysis with SodC-specific antiserum, and loss of catalase activity in MEK14 and MEK6 was confirmed by failure of these two strains to produce bubbles when colonies were flooded with H_2O_2.

Consistent with data reported by other investigators (Sha 1994), introduction of the *katE* mutation into *B. abortus* 2308 did not affect the capacity of this strain to produce wild type spleen infection in BALB/c mice at 1 or 4 weeks post infection (Figure 5). In contrast to previous reports, however, we noted that at 12 weeks post infection the *B. abortus katE* mutant showed highly significant attenuation ($p = 0.0005$) in mice compared to 2308. Also similar to a previous report (Tatum et al. 1992), we noted that the *B. abortus sodC* mutant MEK2 showed a slight but significant level ($p<0.05$) of attenuation in BALB/c mice at 1 and 4 weeks post infection (Figure 5). The attenuation of MEK2 at 12 weeks post infection appeared to be quite pronounced in some mice, but a considerable degree of variability was observed in the numbers of brucellae recovered from the spleens of individual mice infected with this strain. Nevertheless, introduction of the *katE* or *sodC* mutation into RWP11 did not enhance the attenuation of this strain in mice beyond the level of attenuation observed for strains carrying the *katE* or *sodC* mutation alone (Figure 6).

III. Construction of a *B. abortus htrA ctpA* (*htrA prc*) double mutant.

The *Brucella prc* homolog has recently been cloned by Dr. S. M. Halling at the National Animal Disease Center (Halling and Koster, 1999). This gene has been given the designation *ctpA* based on the phylogenetic relatedness of *B. abortus* and *Bartonella henselae* and the fact that the *prc* homolog of this latter bacterium carries the designation *ctpA* (Mitchell and Minnick, 1997). We have received the cloned *B. abortus ctpA* from Dr. Halling and constructed a gene replacement vector designed to replace a 1.2 kb fragment from the *ctpA* coding region with the chloramphenicol resistance gene from p34Cm-2 by allelic exchange. We are presently attempting to use this vector to introduce *ctpA* mutations into *B. abortus* 2308 and RWP11 (*htrA*).

III. Genetic complementation of the *B. abortus bacA* mutant with respect to its ability to resist killing by cultured murine macrophages.

The *in vitro* and *in vivo* properties of the *B. abortus bacA* mutant KL7 were reported in the Final Annual Progress Report for contract DAMD17-94-C-4054 submitted in August of 1998. Two relevant characteristics of this mutant are its accelerated clearance from infected mice and its inability to replicate in cultured murine macrophages (LeVier et al. 1999). Indeed, its clearance profile and immunogenicity in mice suggest that KL7 has considerable potential as a vaccine candidate. Genetic complementation of the bleomycin resistant phenotype of KL7 was accomplished by introducing the *bacA* gene on a pBBR1MCS-based plasmid. Unfortunately, introduction of the *bacA* gene on this plasmid did not complement the sensitivity of this mutant to killing by cultured murine macrophages or its attenuation in mice (data not shown). Attempts to restore the virulence of KL7 in mice and cultured macrophages through the introduction of the *bacA* gene on a plasmid vector with a lower copy number than pBBR1MCS were also been unsuccessful.

As an alternative strategy for genetic complementation of KL7, a wild type version of the *Brucella bacA* gene was introduced into the genome of this mutant by homologous recombination and selection for transformants with the antibiotic resistance profile indicative of a single cross-over event, e.g. ampicillin resistance. Because the *sacB* gene is encoded on the vector integrated in this fashion, selection for sucrose resistance was used to screen for descendants of these transformants in which the *sacB* gene had been deleted through a second recombination event resulting in resolution of their partial diploid state with respect to *bacA*. One strain constructed in this fashion, KL74.3 showed wild type sensitivity to the bleomycin, suggesting that the *bacA* locus had been faithfully reconstructed in this strain. Reconstruction of the *bacA* locus in KL74.3 was subsequently confirmed by Southern blot analysis with *bacA*- and vector-specific probes. As expected, KL74.3 demonstrated wild type resistance to killing by cultured murine macrophages (Figure 7). This established that the defect in macrophage resistance demonstrated by KL7 is a direct consequence of the *bacA* mutation introduced into this strain. The spleen colonization profile of KL74.3 through 12 weeks post infection is presently being compared with that of KL7 and 2308.

IV. Evaluation of *B. abortus hfq* and *bacA* mutants in interferon-γ deficient mice

Previous studies in our laboratory have shown that *B. abortus hfq* and *bacA* mutants are defective in their ability to resist killing by cultured murine macrophages and show accelerated clearance from the spleens and livers of experimentally infected BALB/c mice relative to virulent *B. abortus* 2308 (Robertson and Roop,1999; LeVier et al., 1998; LeVier et al., 1999). Preliminary studies also indicate that the *B. abortus hfq* mutant Hfq3 induces protective immunity in BALB/c mice against subsequent challenge with strain 2308 (G. T. Robertson and M. E. Kovach, unpublished) comparable to that elicited by *B. abortus* strain 19, the live attenuated vaccine instrumental in the virtual elimination of brucellosis from U.S. cattle herds. In order to determine what effect the presence of an intact immune system has on the accelerated clearance profiles of the *B. abortus hfq* and *bacA* mutants in mice, interferon-γ knockout mice derived from the C57BL/6 lineage were infected with *B. abortus* Hfq3 (2308 *hfq*), KL7 (2308 *bacA*) and 2308. The studies were performed in collaboration with Dr. Cynthia Baldwin in the

Department of Veterinary and Animal Sciences at the University of Massachusetts. Previous studies have shown that 2308 replicates to higher than normal numbers in the spleens and livers of these mice and eventually the mice die (or become so obviously sick that they have to be sacrificed) at approximately 6 weeks post infection (M. Parent and C. Baldwin, unpublished). This is in direct contrast to the infection profile of 2308 in wild type C57BL/6 mice, in which chronic infection of the spleen and liver infection is established (e. g. the well-characterized "plateau phase") and no lethality or outward signs of clinical disease are observed. The colonization profile of KL7 in the IFN-γ knockout mice resembled that of 2308 with mice being moribund and having to be sacrificed at 6 weeks post infection (Table 2). Correspondingly, greater than 5 logs of brucellae per spleen were cultured from the spleens of the IFN-γ knockout mice infected with 2308 or KL7 at 6 weeks post infection. In dramatic contrast, the IFN-γ knockout mice infected with Hfq3 remained outwardly healthy throughout the experiment and had essentially cleared their infection at the 6 week time point. These preliminary studies indicate that the integrity of the murine immune system plays a crucial role in the ability of the mouse to clear the *bacA* mutant. On the other hand, the *hfq* mutant appears to be cleared from the murine host independently of a wild type immune response.

V. Construction of a *B. abortus bacA hfq* double mutant.

Although both *B. abortus hfq* and *bacA* mutants are extremely attenuated in mice, both strains elicit strong *Brucella*-specific cellular and humoral immune responses, and immunization of mice with the *B. abortus hfq* mutant Hfq3 provides significant protection against subsequent challenge with the virulent parental strain. Consequently, in an attempt to further stabilize the attenuation of Hfq3 and enhance the utility of this strain as a vaccine candidate, we introduced the *bacA* mutation into Hfq3, resulting in the construction of GR123 (2308 *hfq bacA*). We plan to investigate the *in vitro* and *in vivo* phenotypes of this double mutant in future experiments.

VI. Evaluation of the *in vivo* and *in vitro* phenotypes of a *B. abortus lon clpA* mutant.

ClpP is a serine protease with functions in conjunction with the corresponding chaperones ClpX and ClpA to form the ClpPX and ClpPA complexes, which serve as ATP-dependent stress response proteases (Maurizi, 1992). Previous experiments in our laboratory have shown that ClpPX complex is essential in *B. abortus* (Robertson and Roop, 1998; Robertson et al., 1998), but others have reported that the ClpPA protease complex is not (S. Köhler, personal communication). We have also shown that the other major bacterial cytoplasmic ATP dependent stress response protease, Lon, is dispensable in *B. abortus*, both *in vitro* and *in vivo* (Robertson and Roop, 1998). To examine the phenotypic characteristics of a *B. abortus* strain lacking both ClpPA and Lon, we employed allelic exchange to replace a 0.6 kb fragment of the *clpA* coding region in the *B. abortus lon* mutant GR106, resulting in the construction of the *lon clpA* double mutant GR128. As a control for this experiment we also introduced the *clpA* mutation into *B. abortus* 2308. The genotypes of GR120 and GR128 were confirmed by Southern blot analysis and the presence or absence of ClpA and Lon production in these strains confirmed by Western blot analysis with *Brucella* ClpA- and Lon-specific sera. GR106, GR120 and GR128 all showed significantly increased sensitivity to the antibiotic puromycin and the arginine analog canavanine relative to 2308 (Figure 8). Incorporation of either of these compounds into growing peptide chains during translation results in the intracellular accumulation of incomplete and/or misfolded proteins, and thus the phenotypes of these mutants are consistent with loss of stress response protease activity. More importantly, the *lon clpA* double mutant GR128 showed much greater sensitivity to both puromycin and canavanine than did either the *lon* or *clpA* mutant (Figure 8), strongly suggesting that the biological functions of Lon and ClpA in *Brucella* overlap, at least with respect to their function as stress response proteases. Another interesting component of the phenotypes of the *B. abortus lon* and *clpA* mutants is their sensitivity to H_2O_2 (Figure 8), which also enhanced in the *lon clpA* double mutant GR128.

When the resistance of the *B. abortus lon*, *clpA* and *lon clpA* double mutants to killing by cultured murine macrophages was evaluated, all three mutants displayed defective intracellular

survival and replication compared to 2308 (Figure 9), with the *lon clpA* double mutant showing the most pronounced defect. Correspondingly, the *B. abortus clpA* and *lon clpA* mutants also showed significant attenuation in BALB/c mice (Figure 10).

VII. Genetic complementation of the *B. abortus uvrA* mutant MEK5.

Studies performed under our previous USAMRMC contract (DAMD17-94-C-4054) showed that the *B. abortus uvrA* mutant MEK5 shows significant and stable attenuation in the BALB/c mouse model (Kovach and Roop, 1998). In subsequent studies performed under our present contract, we have shown that introduction of the parental *uvrA* gene into this mutant on a the pBBR1MCS-based plasmid restores wild type resistance to the DNA-damaging agent mitomycin C, but only partially restores the virulence of this strain in mice (data not shown). Consequently, to confirm the genotype-phenotype relationship in MEK5 we will attempt to reconstruct the uvrA locus in this mutant using the *sacB*-based counterselection strategy described previously in Section III of the body of this report.

KEY RESEARCH ACCOMPLISHMENTS

- Discovery of the double mutant nature of *B. abortus* PHE1 and construction and characterization of authentic *B. abortus* and *B. melitensis htrA* mutants.
- Establishing that the *Brucella hfq*, *katE*, *bacA*, *clpA* and *uvrA* are much better targets for the construction of potential vaccine candidates than *htrA*.
- Determining that an intact host immune system appears to required for clearance of the *B. abortus htrA* mutant from mice, but is not required for clearance of the *hfq* mutant. This difference in *in vivo* phenotype could have important implications with respect to the utility of these strains as vaccine candidates.

REPORTABLE OUTCOMES

Manuscripts, abstracts and presentations:

Robertson, G. T., and R. M. Roop II. 1999. The *Brucella abortus* host factor I (HF-I) contributes to stress resistance during stationary phase and is a major determinant of virulence in mice. Mol. Microbiol. (*in press*) - **manuscript**

Robertson, G. T., A. Teixera-Gomes, A. Cloeckart, M. S. Zygmunt, and R. M. Roop II. "The essential nature of the *Brucella clpP* and *clpX* gene products." Presented at the 51st Annual Brucellosis Research Conference, November 7, 1998. - **presentation**

Robertson, G. T., A. Teixeira-Gomes, A. Cloeckart, M. S. Zygmunt, and R. M. Roop II. 1998. The *Brucella abortus* ClpP and ClpX proteins are not under heat shock control and appear to be essential for viability. Proc. 79th Annu. Conf. Res. Work. Anim. Dis., Abstr. 5, p. 41. – **abstract**

LeVier, K., R. W. Phillips, G. T. Robertson, A. Reisenauer, R. W. Wright, A. E. Jensen, G. C. Walker, L. A. Shapiro, and R. M. Roop II. 1999. Developmental genes in *Brucella* and their potential role in virulence in the mammalian host. Abstr. 43rd Annu. Wind River Conf. Proc. Biol., Abstr. 14, p. 28. - **abstract**

Robertson, G. T., and R. M. Roop II. 1999. Maintenance of stationary phase is required for establishment of chronic infection by *Brucella abortus* in the murine model. Abstr. 43rd Annu. Wind River Conf. Proc. Biol., Abstr. 16, p. 32. - **abstract**

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Gregory T. Robertson – Postdoctoral Fellow, Infectious Disease Research, Lilly Research Laboratories – scheduled to start in November 1999

CONCLUSIONS

The *Brucella hfq*, *bacA*, *katE*, *clpA* and *uvrA* loci appear to be much better targets for the construction of potential vaccine candidates than *htrA*.

I. *hfq*

As described in previous reports, the *B. abortus hfq* gene product appears to be required for maintenance of the stationary phase growth state (Robertson and Roop, 1999). More importantly, the capacity to maintain this physiologic state seems to be essential for long term survival in host macrophages. Because the *B. abortus hfq* mutant Hfq3 shows increased susceptibility to at least three different environmental stresses relevant to the phagosomal compartment – H_2O_2, acidic pH and nutrient deprivation – it is difficult to precisely define the biological basis for their attenuation in mice. Indeed, this phenotype is likely pleiotropic. Nevertheless, demonstrating the importance of the stationary phase growth state for the chronic persistence of brucellae in host macrophages represents an important contribution to our understanding of the basic biology of *Brucella* infections.

Not only does the *B. abortus hfq* mutant Hfq3 show significant attenuation and accelerated clearance in mice, but infection with this strain induces strong humoral and cellular immune responses which provide a significant degree of protection against subsequent challenge with virulent *B. abortus* 2308. In this regard, it is particularly interesting that the attenuation of Hfq3 remains stable in IFN-γ deficient mice. These findings suggest that this mutant will be unlikely to revert to an immunocompromised host. They also raise the possibility, however, that this strain may be too attenuated to induce optimal protective immunity in some hosts.

II. *bacA*

Like the *B. abortus hfq* mutant, the *bacA* mutant KL7 shows accelerated clearance from experimentally infected mice and induces strong *Brucella*-specific humoral and cellular immune responses (LeVier et al., 1998; LeVier et al., 1999). The capacity of this strain to provide protection against subsequent challenge with virulent *B. abortus* 2308 has yet to be determined,

however. The basis for the attenuation of KL7 in mice is presently unknown. Based on the proposed function of its homolog in *Rhizobium meliloti* (Glazebrook et al., 1993), however, we suspect that this gene product is required for a programmatic change in gene expression which occurs in the brucellae during the early stages of their adaptation to the phagosomal compartment. This change in gene expression most likely results in a dramatic shift in the metabolic state of the brucellae which may resemble that which occurs when the free-living rhizobia switch to their intracellular symbiotic state.

The wild type virulence of KL7 in the IFN-γ deficient mice indicates that an intact immune system is required for clearance of this strain from the host. Obviously, this is an important consideration with respect to the utility of this mutant as a vaccine candidate. On the one hand, this requirement for an optimal host immune response may be beneficial in terms of the duration and intensity of the host immune responses elicited as the result of inoculation with KL7. On the other hand, this strain may retain too much residual virulence to be useful in immunocompromised hosts.

III. *Brucella hfq bacA, hfq purE* and *bacA purE* double mutants

The difference observed in the virulence of the *B. abortus bacA* and *hfq* mutants in the IFN-γ deficient mice also indicates that the basis for the attenuation of these strains in normal mice is probably different. Therefore, it is quite possible that combining these two mutations will not result in further attenuation beyond that already observed for Hfq3 and KL7 in mice. The obvious benefit of combining these mutations is that this would dramatically reduce the chances for reversion to virulence in the host. *Brucella hfq bacA* double mutants should also lack the residual virulence observed for KL7 in IFN-γ deficient mice. As outlined in the body of this report, we have constructed a *B. abortus hfq bacA* mutant, but this strain has not been characterized as yet.

Similar benefits may also arise from combining either the *hfq* or *bacA* mutation with the *purE* mutation present in the *B. melitensis* strain presently being examined as a vaccine candidate by workers at the Walter Reed Army Institute of Research (Crawford et al., 1996). To accomplish this, we intend to pursue a collaborative arrangement with Col. David Hoover and Dr. Mikeljon Nikolich at WRAIR to construct and characterize *Brucella hfq purE* and *bacA purE* mutants *in vitro* and in the mouse model. Drs. Kristin LeVier and Graham Walker of the Department of Biology at the Massachusetts Institute of Technology will also be involved in the construction and characterization of the *Brucella bacA purE* mutants.

IV. *katE, clpA* and *uvrA*

Although our future studies will concentrate primarily on the *Brucella hfq* and *bacA* mutants, our experimental results indicate that the *katE, clpA* and *uvrA* also offer attractive targets for the construction of novel vaccine candidates. Defining the contribution of the corresponding gene products to the virulence of the brucellae will also provide important insight into the biology of *Brucella* infections in the host.

a. *katE*

KatE is the sole catalase described for *Brucella* to date (Sha et al., 1994), and the extreme sensitivity of the *B. abortus katE* mutant MEK6 to H_2O_2 *in vitro* supports the singular nature of this important primary antioxidant. Moreover, the onset of accelerated clearance of MEK6 from the spleens of mice relative to 2308 at some point following 4 weeks post infection suggests that the brucellae are subjected to an oxidative stress during this period and that KatE is required to resist this assault. Previous studies have shown that optimal induction of cell-mediated immunity occurs during this same period in mice (Araya et al., 1989) and its seems likely that the accelerated clearance of MEK6 from mice coincides with the development of fully activated macrophages which would be expected to have considerable oxidative killing capacity. These findings support earlier studies demonstrating the importance of the oxidative killing pathways of host macrophages for the elimination of intracellular brucellae (Jiang et al., 1993).

b. *clpA*

The results of our studies with the *B. abortus clpA* mutant GR120 and the *lon clpA* double mutant GR128 strongly suggest that the ClpAP complex functions as a stress response protease and that the biological functions of the *Brucella* Lon and ClpAP overlap in at least in some aspects. The spleen colonization profiles of these strains in mice also suggest that Lon and the ClpAP complex interact to perform some biological function that is critical for wild type virulence in the murine host. Based on the *in vitro* phenotypes of GR106, GR120 and GR128 one possible contributor to the attenuation of these strains is their susceptibility to oxidative killing, which seems to be enhanced when the two mutations are combined. These results are puzzling because previous biochemical and genetic studies have clearly shown that the prokaryotic ATP-dependent proteases such as Lon and ClpP do not participate in the degradation of oxidatively damaged proteins (Davies and Lin, 1988). Clearly, further evaluation of the *in vitro* and *in vivo* properties of these strains will be required to gain a clearer picture of the basis for the attenuation of the *B. abortus clpA* and *lon clpA* mutants in mice.

c. *uvrA*

Previous studies in our laboratory have shown that the *B. abortus uvrA* mutant MEK5 shows significant and stable attenuation in the mouse model (Kovach and Roop, 1998). As detailed in the Final Report for our previous USAMRMC contract (DAMD17-94-C-4054), however, we are presently unable to define the basis for this attenuation UvrA plays a major role in the repair of DNA damage in bacterial cells (Friedberg et al., 1995). Although the *B. abortus uvrA* mutant shows increased sensitivity to DNA damaging agents *in vitro*, we see no evidence for an increased sensitivity to either Mode I or Mode II type oxidative killing (Imlay and Lin, 1988). This is particularly intriguing because exposure to ROIs would be the most likely environmental stress relevant to the phagosomal compartment expected to produce DNA damage. Indeed, Mode I type oxidative damage to the DNA of intracellular pathogens has been proposed by some investigators as a major killing mechanism of host macrophages (Buchmeier et al., 1995). Thus, further evaluation of the *in vitro* and *in vivo* phenotypes of the *B. abortus uvrA* mutant will be required before we can define the basis for its attenuation in the murine host.

V. *ctpA*

The *Brucella prc* homolog, designated *ctpA*, has been cloned (Halling and Koster, 1999) and a *B. abortus ctpA* mutant is presently being characterized in vitro and in vivo (S. M. Halling, personal communication). Because there is both genetic and biochemical evidence suggesting overlapping function between *htrA* and *ctpA* (*prc*) in other bacteria (Davies and Linn, 1988; Bäumler et al., 1994), we are presently attempting to construct a *B. abortus ctpA* and an isogenic *htrA ctpA* double mutant to determine the extent of this overlap in function, if any, in *Brucella*. We are particularly interested in what effect combining these mutations will have on the capacity of the brucellae to withstand ROI-mediated killing *in vitro* and *in vivo*. These particular genetic studies are funded by a grant from the USDA. However, if the *B. abortus htrA ctpA* mutant shows synthetic enhancement of its ROI-sensitive phenotype and significant and stable attenuation in mice, then it will be interesting to construct a *B. melitensis htrA ctpA* mutant and examine its virulence in the mouse model and potential as a vaccine candidate.

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APPENDIX A. FIGURES AND TABLES FOR THE ANNUAL REPORT.

Figure 1. Diagram of the placement of the kanamycin resistance gene in *B. abortus* PHE1 (2308 *htrA cycL*), RWP11 (2308 *htrA*), RWP501 (2308 *cycL*) and RWP116 (2308 *htrA cycL*). Boxes containing the designations of *Brucella* mutants demonstrate the location of the kanamycin resistance gene in each mutant. The approximate location of restriction enzyme recognition sites used in the construction of these mutants is also shown. The stick arrow at the end of the *cycL* gene indicates the position of the σ^E type promoter sequence of *htrA*.

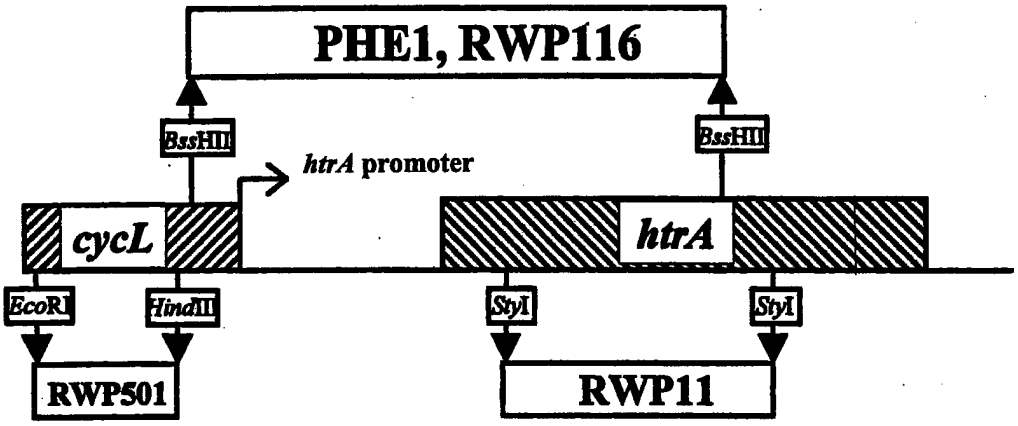

Table 1. Sensitivity of *B. abortus* 2308, RWP11 (2308 *htrA*) and RWP11(pRIE1) to killing by hydrogen peroxide and puromycin in a disk sensitivity assay.

Strain	H_2O_2	Puromycin
2308	49.88 ± 2.76[a]	18.3 ± 2.51
RWP11	62.66 ± 2.97**	26.0 ± 1.41*
RWP11 (pRIE1)	48.16 ± 3.34	20.0 ± 1.0

[a]Zone of inhibition in mm
*$p<0.05$; **$p<0.01$

Figure 2. Intracellular survival of IgG opsonized *B. abortus* 2308, RWP11 (2308 *htrA*), RWP501 (2308 *cycL*) and RWP116 (2308 *htrA cycL*) after 24 hours within cultured murine peritoneal macrophages. Error bars indicate standard deviation. Significance values - *$p<0.01$ for comparison of RWP11, RWP501 or RWP116 with 2308.

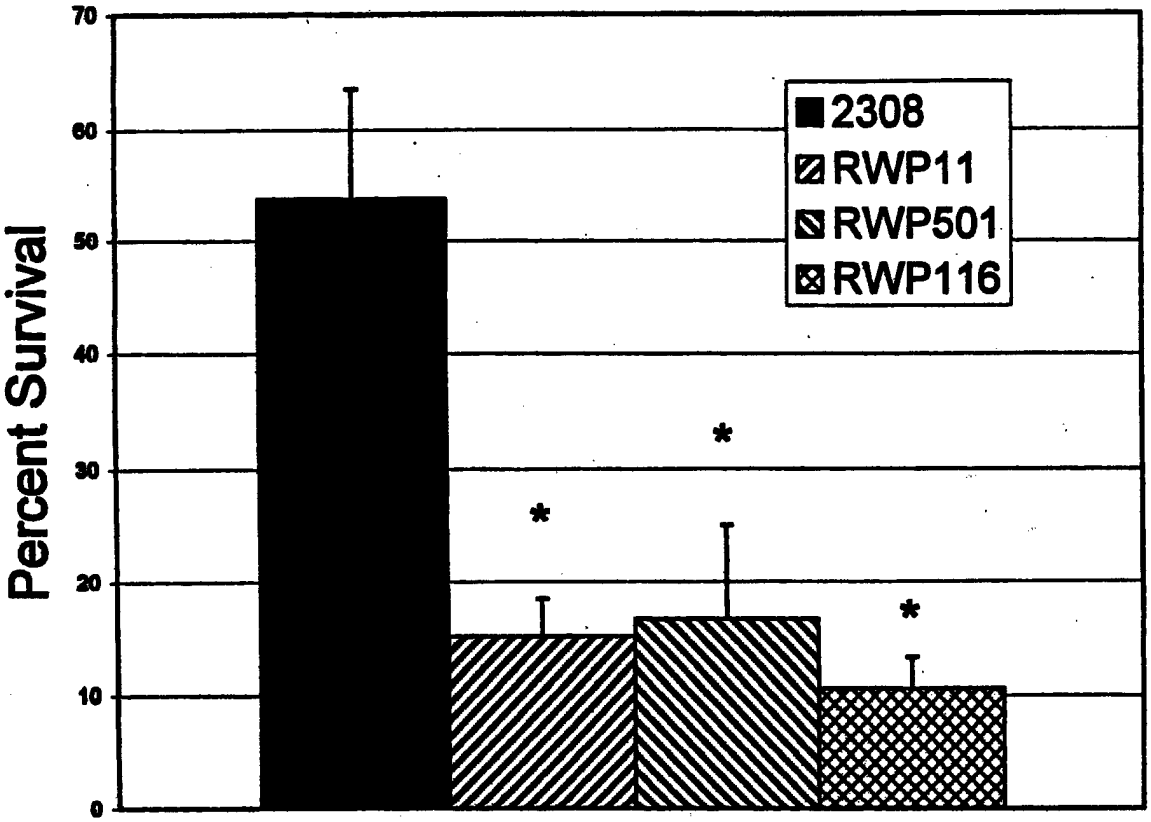

Figure 3. Survival of *B. abortus* 2308, RWP11 (2308 *htrA*), RWP501(2308 *cycL*) and RWP116 (2308 *htrA cycL*) after 120 minutes incubation in the presence of cultured murine neutrophils. Error bars indicate standard deviation. Significance values - **p*<0.05 and ***p*<0.01 for comparison of RWP11, RWP501 or RWP116 with 2308.

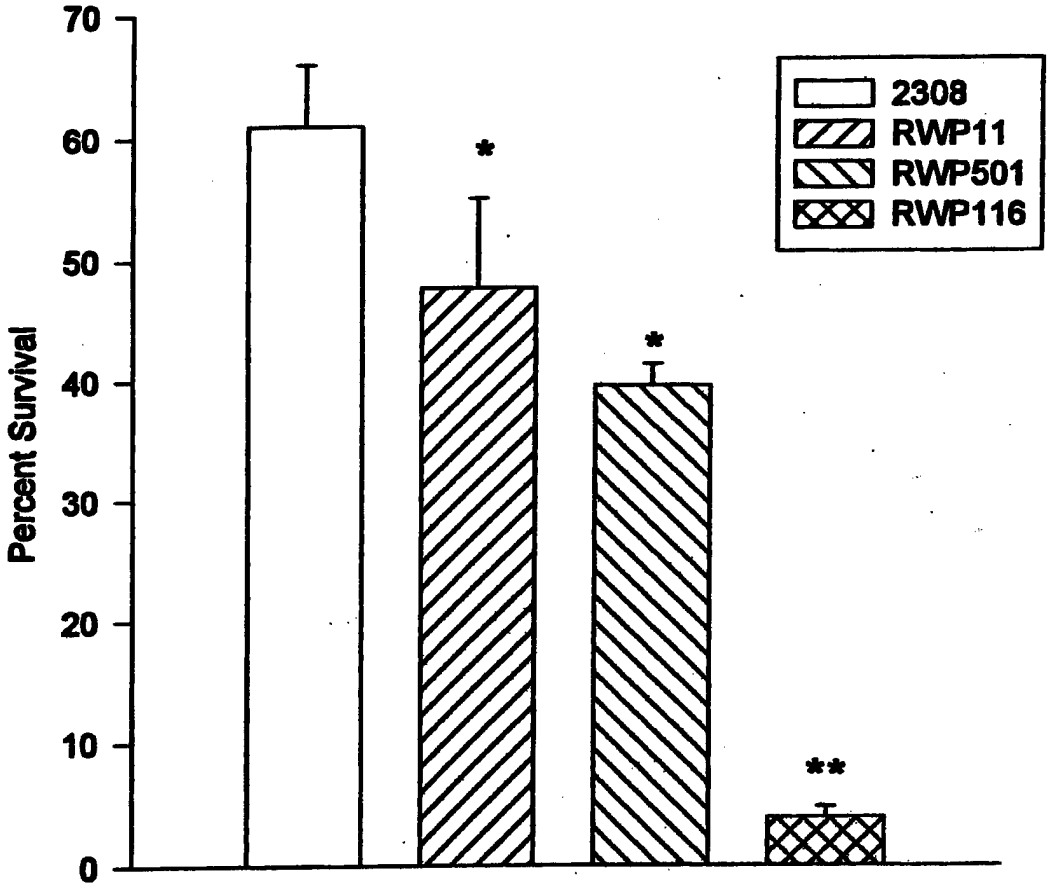

Figure 4. Spleen colonization of BALB/c mice by *B. abortus* 2308, RWP11 (2308 *htrA*), RWP501 (2308 *cycL*), and RWP116 (2308 *htrA cycL*). Error bars indicate standard deviation. Significance values – **p*<0.05, ***p*<0.01 for differences between RWP116 and 2308. Colonization levels for *B. abortus* 2308 at 3 weeks post infection are not shown due to contamination of the culture.

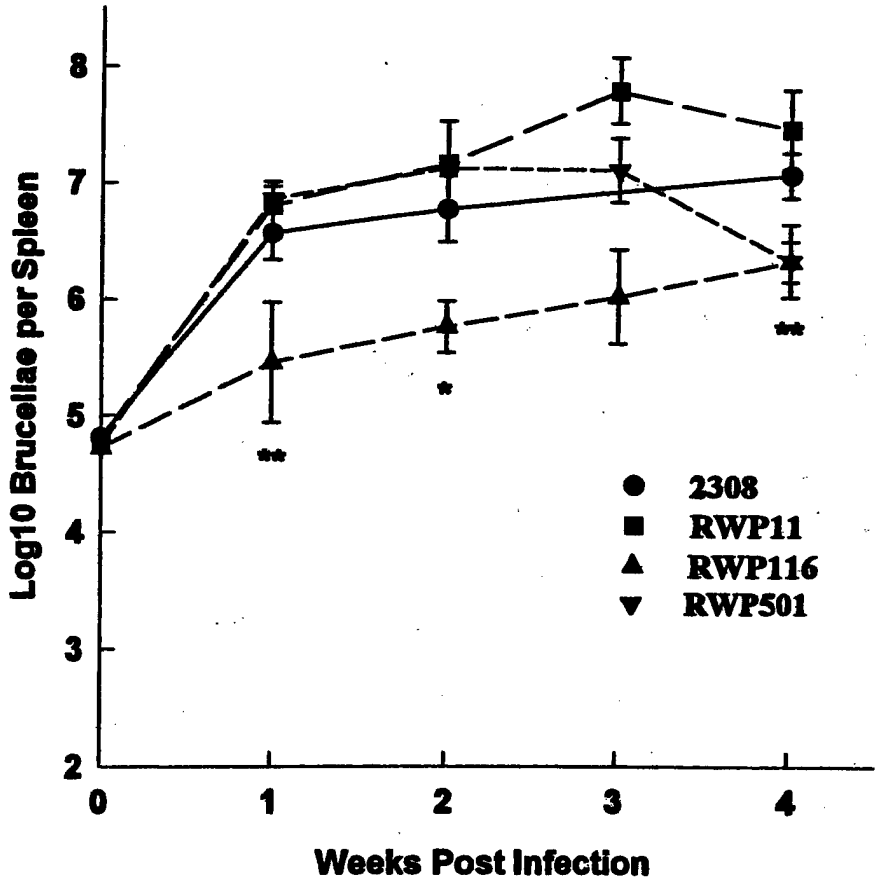

Figure 5. Spleen colonization of BALB/c mice by *B. abortus* 2308, MEK2 (2308 *sodC*), MEK6 (2308 *katE*) and MEK10 (2308 *sodC katE*). Error bars indicate standard deviation. Significance values – $p<0.05$ for MEK2 and MEK10 compared to 2308 at 12 weeks post infection; $p= 0.0002$ for MEK6 compared to 2308 at 12 weeks post infection.

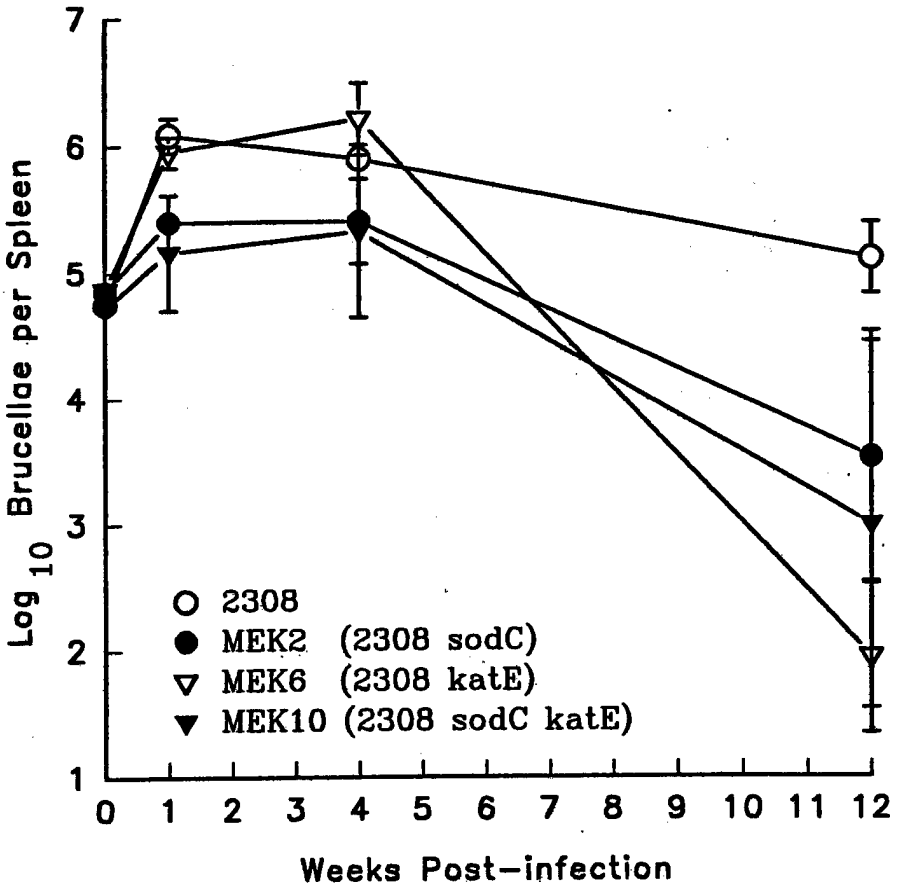

Figure 6. Spleen colonization of BALB/c mice by *B. abortus* 2308, RWP11 (2308 *htrA*), MEK9 (2308 *htrA sodC*) and MEK14 (2308 *htrA katE*). Error bars indicate standard deviation. Significance values - $p<0.005$ for MEK14 compared to 2308 and RWP11.

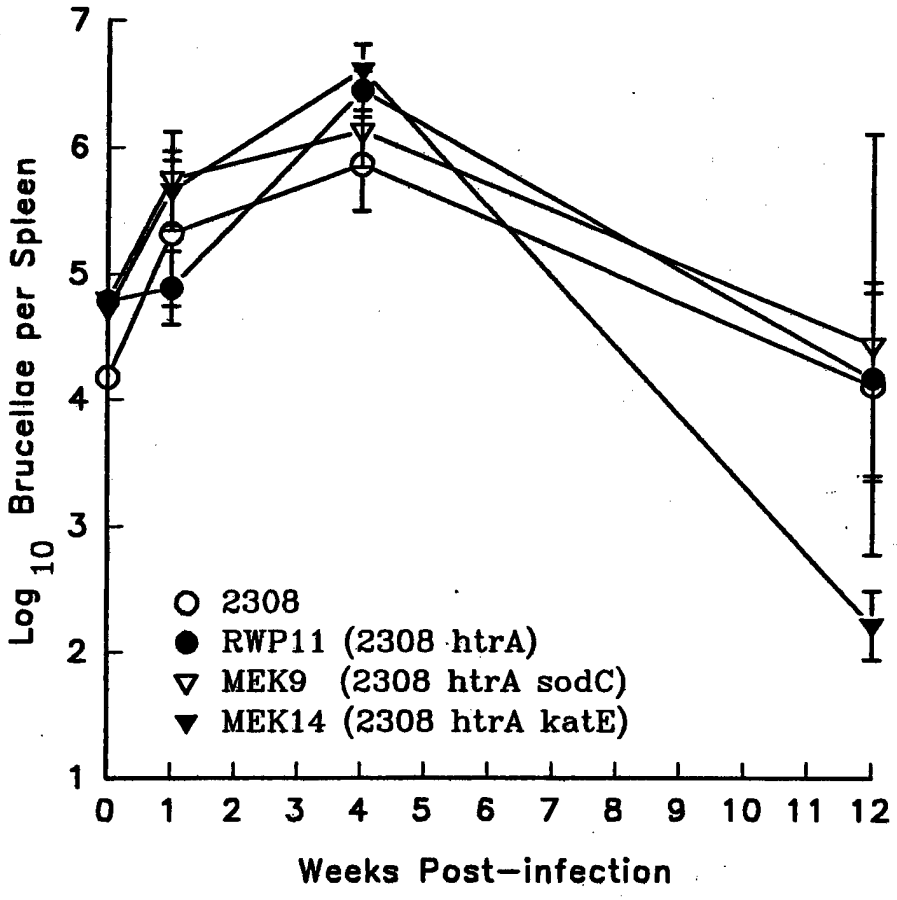

Figure 7. Intracellular survival of IgG-opsonized *B. abortus* 2308, KL7 (2308 *bacA*) and KL74.3 (KL7 *bacA*$^-$ → *bacA*$^+$) in cultured murine macrophages. $p<0.05$ for comparisons of 2308 and KL74.3 with KL7 at all time points.

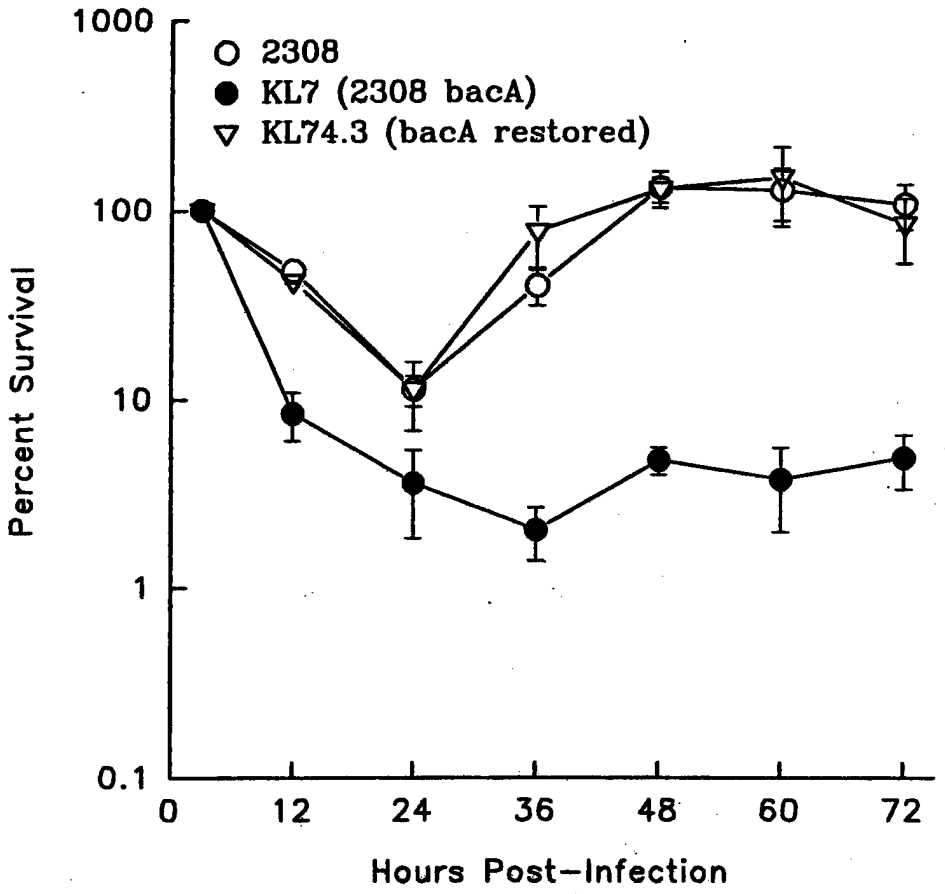

Table 2. Spleen colonization profiles of *B. abortus* 2308, KL7 (2308 *bacA*) and Hfq3 (2308 *hfq*) in IFN-γ deficient mice at 4 and 6 weeks post infection. The data presented represents mean values determined for 5 animals in each experimental group.

4 weeks post infection

	2308[a]	KL7[a]	Hfq3
Log cfu/spleen	5.3 ± 0.35	4.68 ± 0.14	2.58 ± 2.47
Spleen weight (in mg)	874.8 ± 90.9	745 ± 94.1	525 ± 422.2

6 weeks post infection

	2308[a]	KL7[a]	Hfq3
Log cfu/spleen	6.35 ± 0.14	5.4 ± 0.18	---[b]
Spleen weight (in mg)	783 ± 273	1018 ±113	124 ± 41

[a]Mice infected with 2308 and KL7 showed signs of illness (lethargy, matted hair, wasting, tail abscesses) at both 4 and 6 weeks post infection, with these signs becoming so severe that the mice became moribund and were sacrificed at the latter time point.

[b]No brucellae were isolated from the spleens 4 of the 5 mice in this group and 2 organisms were recovered from the spleen of the remaining mouse.

Figure 8. Sensitivity of *B. abortus* 2308, GR106 (2308 *lon*), GR120 (2308 *clpA*) and GR128 (2308 *lon clpA*) to killing by a) H_2O_2, b) puromycin and c) canavanine in disk sensitivity assays. Error bars denote standard deviation. Significance - $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ for comparisons of GR106, GR120 and GR128 with 2308; $^{+}p<0.05$, $^{++}p<0.01$ for comparisons of GR120 and GR128 with GR106; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ form comparisons of GR106 and GR128 with GR120.

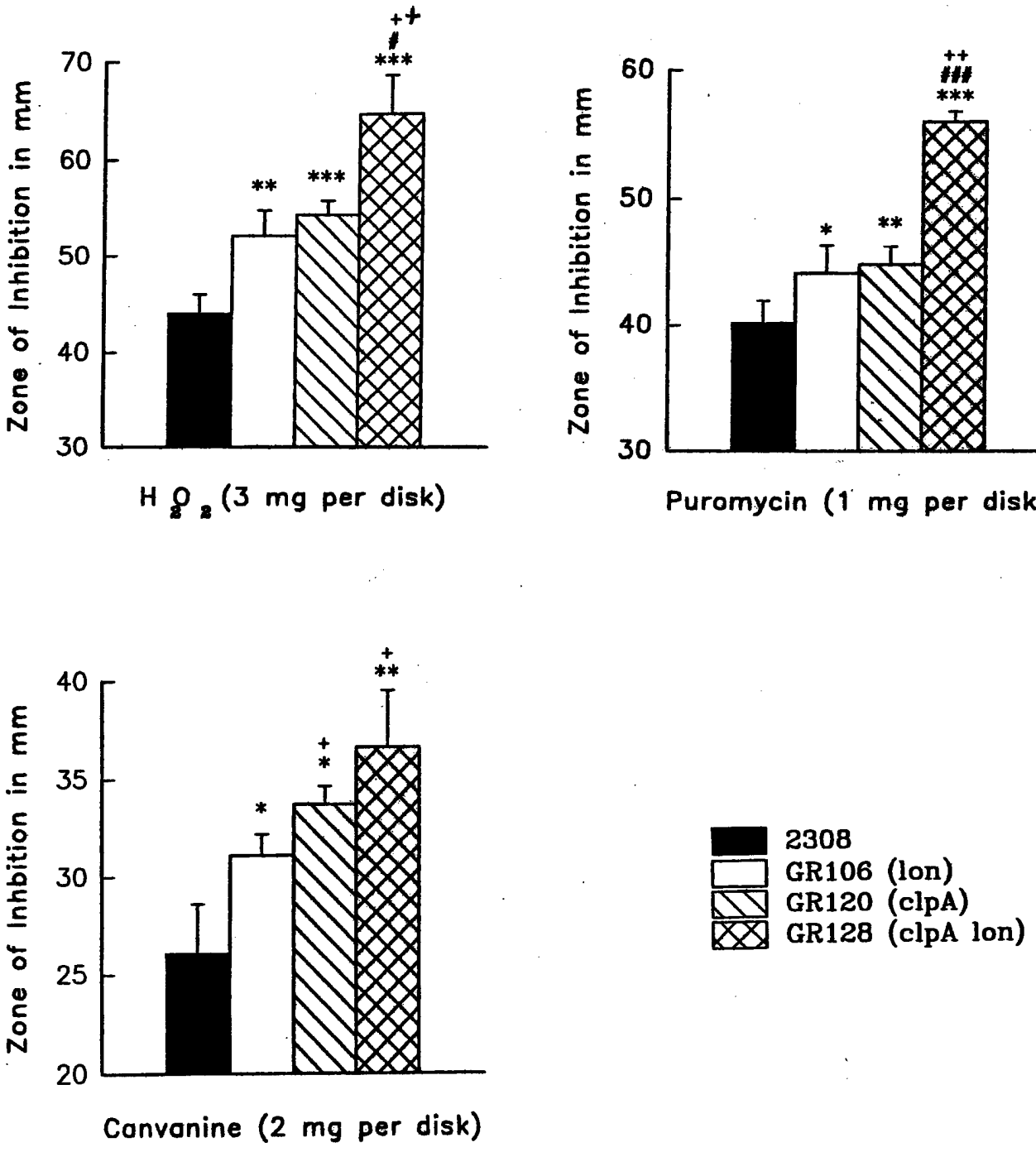

Figure 9. Intracellular survival of IgG-opsonized *B. abortus* 2308, GR106 (2308 *lon*), GR120 (2308 *clpA*) and GR128 (2308 *lon clpA*) in cultured murine peritoneal macrophages. $p<0.05$ for comparisons of GR106, GR120 and GR128 with 2308 at 48 and 60 hours post infection.

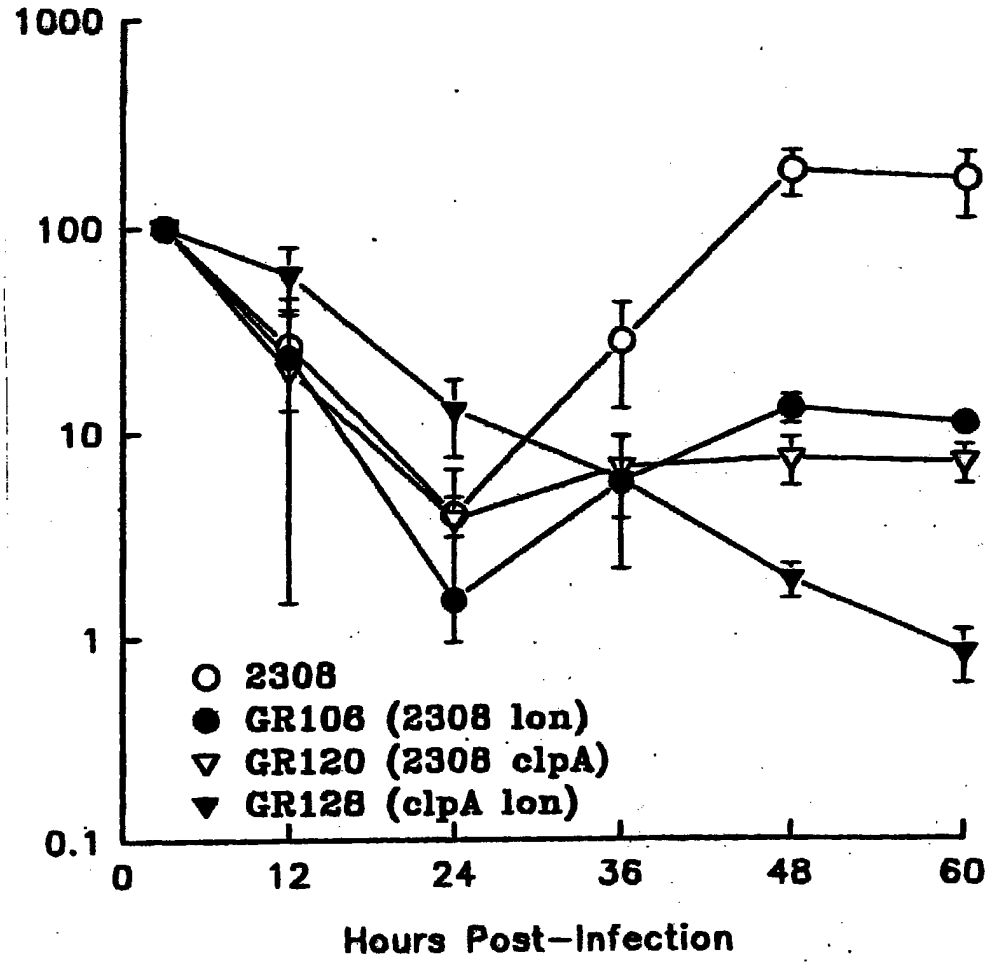

Figure 10. Spleen colonization of BALB/c mice by *B. abortus* 2308, GR106 (2308 *lon*), GR120 (2308 *clpA*) and GR128 (2308 *lon clpA*). Error bars indicate standard deviation. $p < 0.01$ for comparisons of GR128 with 2308 at 1 week post infection and for comparisons of GR120 and GR128 with 2308 at 4 weeks post infection.